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Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population

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Abstract

The existence of transgenic hybrids resulting from transgene escape from genetically modified (GM) crops to wild or weedy relatives is well documented but the fate of the transgene over time in recipient wild species populations is still relatively unknown. This is the first report of the persistence and apparent introgression, i.e. stable incorporation of genes from one differentiated gene pool into another, of an herbicide resistance transgene from *Brassica napus* into the gene pool of its weedy relative, *Brassica rapa*, monitored under natural commercial field conditions. Hybridization between glyphosate-resistant [herbicide resistance (HR)] *B. napus* and *B. rapa* was first observed at two Québec sites, Ste Agathe and St Henri, in 2001. *B. rapa* populations at these two locations were monitored in 2002, 2003 and 2005 for the presence of hybrids and transgene persistence. Hybrid numbers decreased over the 3-year period, from 85 out of ~200 plants surveyed in 2002 to only five out of 200 plants in 2005 (St Henri site). Most hybrids had the HR trait, reduced male fertility, intermediate genome structure, and presence of both species-specific amplified fragment length polymorphism markers. Both F_1 and backcross hybrid generations were detected. One introgressed individual, i.e. with the HR trait and diploid ploidy level of *B. rapa*, was observed in 2005. The latter had reduced pollen viability but produced ~480 seeds. Forty-eight of the 50 progeny grown from this plant were diploid with high pollen viability and 22 had the transgene (1:1 segregation). These observations confirm the persistence of the HR trait over time. Persistence occurred over a 6-year period, in the absence of herbicide selection pressure (with the exception of possible exposure to glyphosate in 2002), and in spite of the fitness cost associated with hybridization.

Keywords: *Brassica napus*, *Brassica rapa*, gene flow, genetically modified crops, hybridization, transgene escape

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Introduction

Confinement of transgenes is often an environmental as well as an agricultural issue. As transgenic crop varieties are increasingly grown in commercial agriculture (James 2006), the potential for transgene escape to related wild and/or weedy relatives is likely to increase through the formation of transgenic hybrid populations (Warwick *et al*. 1999, 2004; Ellstrand 2001; Snow 2002; Stewart *et al*. 2003).

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One possible negative consequence associated with the inadvertent production of transgenic hybrids is an increase in fitness and invasiveness of weedy related species. Although crops and weeds have exchanged genes for centuries, genetic engineering introduces genes that confer novel or enhanced fitness-related traits into ecosystems, whereas crop genes often confer limited fitness benefits. These novel genes can come from various sources including distant plant species, bacteria, viruses, animals, artificial synthesis, etc., and are introduced into many diverse types of crops, each with its own specific potential to outcross (Snow 2002). Since most currently commercialized transgenic or

genetically modified (GM) crops have wild or weedy relatives in all or parts of their range (Ellstrand *et al*. 1999), the potential for gene flow is real but will vary according to geographical location. In the western USA, natural hybridization and introgression has been reported with herbicide-resistant *Triticum aestivum* L. (wheat) and sympatric populations of its weedy relative *Aegilops cylindrica* Host. (jointed goatgrass; reviewed in Hegde & Waines 2004). Similarly in the USA, hybrid formation and introgression is known for cultivated sunflower and its wild relatives (Whitton *et al*. 1997; Linder *et al*. 1998).

Transgenic *Brassica napus* L. is of particular concern as the crop is produced worldwide and has a high potential for hybridization with several sexually compatible crops and wild relatives present in most *B. napus*-growing areas. The crop has high interplant outcrossing rates (averaging 30%), and is both insect- and wind-pollinated with pollen flow recorded up to 1–3 km from the crop (reviewed in Warwick *et al*. 2004). Transgenes can escape by both pollen and seed as *B. napus* can form a persistent seed bank, producing volunteer crop populations in subsequent crops (reviewed in Hall *et al*. 2005; Légère 2005; Beckie *et al*. 2006).

Numerous studies (Jørgensen & Andersen 1994; Jørgensen *et al*. 1996; Landbo *et al*. 1996; Halfhill *et al*. 2001, 2002, 2004; Hansen *et al*. 2001, 2003;Warwick *et al*. 2003; Wilkinson *et al*. 2003; Chèvre *et al*. 2004) have indicated the potential for hybridization and introgression between *B. napus* (allotetraploid, AACC genome, 2*n* = 38) and its wild weedy relative *Brassica rapa* (diploid, AA genome, 2*n* = 20), suggesting that *B. napus* transgenes could be easily introduced into weedy *B. rapa* populations. *B. rapa* is a major weed in many countries and many crops (reviewed in Chèvre *et al*. 2004; Hall *et al*. 2005). It has a limited distribution as an agricultural and/or ruderal weed in Canada but is sympatric with *B. napus*-growing areas in several regions of Québec (Simard *et al*. 2006). It is an obligate outcrosser and has overlapping flowering periods with *B. napus*. Seed dormancy is characteristic of weed populations of *B. rapa* (Hall *et al*. 2005), which could allow early generation hybrids to persist in the soil seed bank.

We first detected natural transgenic *B. rapa* × *B. napus* F_1 hybrids under field conditions in 2001 in two Québec populations of weedy B. *rapa* (St Henri, Ste Agathe), where transgenic *B. napus* had been previously cultivated (Warwick *et al*. 2003). This was the first documented case of transgene movement into a natural weed population from a commercial transgenic crop. All F_1 hybrids were confirmed by the presence of the herbicide resistance (HR) trait, and species-specific AFLP (amplified fragment length polymorphism) markers from both parents. These hybrids were morphologically similar to *B. rapa*, had reduced pollen viability (~55%), and were triploid (AAC, 2*n* = 29). Hybridization rates varied markedly between the St Henri population (0.023%) and the Ste Agathe population (13.6%) (Warwick *et al*. 2003). Recent field surveys confirmed that F_1 hybrid production between these two species is common and to be expected when the two species co-occur, as the presence of HR F_1 hybrids was detected in all instances where *B. rapa* was found growing in *B. napus* field borders (Simard *et al*. 2006). In addition, 5% of hybrids grown in the greenhouse were obvious selfers (a *B. napus* trait). Hybridization rates were variable (5.8% ± 2.4 SE) and generally decreased as conspecific *B. rapa* density increased (Simard *et al*. 2006).

Stable introgression through the formation of backcross (BC) generations is dependent on F_1 hybrid fitness, i.e. growth vigour, fertility, and ability to set viable seed and persist in the seed bank. First generation hybrids are often genetically unstable with reduced fertility, features that must be overcome in subsequent backcross generations in order for stable introgression to occur. Previous studies have shown that an HR transgene can be passed between *B. napus* and *B. rapa* and be expressed in successive generations (Mikkelsen *et al*. 1996; Metz *et al*. 1997; Snow *et al*. 1999; Ammitzbøll *et al*. 2005). Genetic studies of transgenic hybrids have also indicated that after one backcross generation, the ploidy of the BC1F1 generation (as assessed by nuclear DNA content) shifted towards that of *B. rapa* (Halfhill *et al*. 2002). In subsequent backcross generations (BC_2F_1 and BC_2F_2), the trend toward the loss of *B. napus* genetic material continued and ploidy level was indistinguishable from that of the diploid *B. rapa* parental species. The diploid composition was stable after the intermating of BC_2F_1 individuals (Halfhill *et al*. 2003), demonstrating that a stable transgenic diploid population can be reached after hybridization and two generations of backcrossing. Snow *et al*. (1999) also reported stable transgenic diploid *B. rapa* plants after three backcross generations.

Monitoring for the persistence and stability of transgenic traits in wild populations is ultimately needed to assess long-term environmental impact. The likelihood that transgenes will persist in wild populations of a weedy relative can be determined by tracking the amount of hybridization and characterizing hybrids occurring naturally over time within wild populations where an initial hybridization event has occurred. The objective of this study was to monitor transgene persistence in *B. rapa* populations over time at the two sites where transgene escape from commercial fields of glyphosate-resistant *B. napus* had been previously documented (Warwick *et al*. 2003). Tools used to detect and characterize hybrids included: presence/absence of the glyphosate-resistance (HR) trait, ploidy level, pollen viability, and presence/absence of crop-specific AFLP molecular markers.

Materials and methods

The two *Brassica rapa* populations, QC-9039 from Ste Agathe de Lotbinère (46E23′N, 71E25′W) and QC-9047 from St Henri de Lévis (46E42′N, 71E04′W) described in Warwick

Table 1 History of agronomic practices used at the sites where the two *Brassica rapa* × *B. napus* hybrid populations were found

Site	Year	Years after contact with HR crop	Crop	Herbicide
St Henri	2000	0	*B. napus* HR*; first time canola grown in this field	Glyphosate
	2001	1	Sweet maize	Metolachlor–preseeding incorporated + Bentazon/atrazine-early postemergence
	2002	2	Pumpkin	Glyphosate-directed postemergence
	2003	3	Sweet maize	Metolachlor–preseeding incorporated + Bentazon/atrazine-early postemergence
	2004	4	Barley	Thifensulfuron/tribenuron postemergence MCPA amine-postemergence
	2005	5	Sweet maize	Metolachlor–preseeding incorporated + Bentazon/atrazine-early postemergence
Ste Agathe	2001	0	*B. napus* HR*; first time canola grown in this field	Glyphosate
	2002	1	Barley	Thifensulfuron/tribenuron postemergence
	2003	2	Barley	Dicamba/MCPA–postemergence
	2004	3	Barley	Thifensulfuron/tribenuron postemergence
	2005	4	*B. napus* (hybrid seed crop)	Weed population on field edge ploughed

*HR, glyphosate-resistant transgenic crop.

et al. (2003) were monitored in 2002 (St Henri site only), 2003, and 2005 to determine the fate of the HR transgene under normal agro-environmental conditions. At both sites, the *B. rapa* populations were located along the field margins and less than 1 m into the crop area. A glyphosate-resistant canola crop was grown at the Ste Agathe site in 2001, and at the St-Henri site in 2000; in both cases, this was the first time that canola had been grown in these two fields (Warwick *et al*. 2003). The history of agronomic practices used at the two sites is given in Table 1. Glyphosate-resistant canola was not grown in any adjacent fields. Glyphosate was only reapplied in 2002 at the St Henri site, and potentially selected *Brassica napus* volunteers and some (but not all) hybrid individuals as most of the *B. rapa* population occurred at the field border. All other herbicide applications effectively controlled the *B. rapa* weeds and the *B. napus* volunteers located in the subsequent field crops at both sites (sweet corn or barley), but left the field borders weedy. In 2005, the Ste Agathe population was destroyed because the field border was ploughed. In 2003, all 69 of the individuals in the Ste Agathe population were sampled. The St Henri *B. rapa* population was much larger (> 1000 plants), and individuals (~200 samples per year) were sampled randomly along two edges of the original *B. napus* field over a distance of ~100 m, including the field area from which the first F_1 hybrid seed (Warwick *et al*. 2003) had been collected. The fields were inspected for plants with morphology similar to *B. rapa* and/or *B. napus*. These plants were tagged, and leaf tissue and flowers collected from each plant in July and seed pods in August. At each site, samples were collected from *B. napus* volunteers growing in parts of the field where *B. rapa* did not occur. Reference populations included *B. napus*, 45A51, a transgenic HR glyphosate-resistant *B. napus* cultivar, and *B. rapa*, QC-2975, a wild *B. rapa* population collected in 1988 at Waterville, Québec (45°16′N, 71°54′W) from the germplasm collection AAFC-ECORC, Ottawa. Five individuals from each control population were screened with each year's field samples to ensure reproducibility of species-specific AFLP markers. Progeny were grown from three putative hybrid/introgressed plants collected in 2005 from the St Henri population. All plants were scored for the HR trait (HR+/HR–), presence of species-specific AFLP markers from both parental species, pollen viability, and ploidy.

Herbicide resistance

Leaf tissue was tested using commercially available Trait RUR Lateral Flow Test strips (Strategic Diagnostics). The RUR trait kit detects the CP4 EPSPS protein produced by the HOR gene, a gene derived from *Agrobacterium* sp. strain CP4 and incorporated into glyphosate-resistant *B. napus*. A small piece of leaf tissue, about 50 mg wet weight, was added to a tube with 2 ceramic beads and 2 mL of water. The samples were ground using a Fast Prep BIO101 shaker. A test strip was added and results recorded as either positive (i.e. presence of two bands, a control band that tracks the capillary action and a second band that indicates the protein) or negative (i.e. control band only) within 5 min.

Ploidy

Ploidy levels were inferred from flow cytometric values using a Partec PA-I Flow cytometer (Partec GmbH) which measures relative amounts of cellular DNA (Dolezel 1991). About 0.5–1 cm^2 of leaf material was chopped with 0.5 mL of lysis buffer (solution A, high resolution plant DNA-kit, Partec) in a Petri dish using a razor blade. Following filtration through a 50 μm nylon mesh filter (Celltrics, Partec), ~2 mL of the staining solution (solution B, Partec), containing DAPI was added. The sample was gently stirred for 1 min and the analysis was performed using a Partec PA-I equipped for UV light excitation and blue light emission (100 W mercury HBO lamp, Partec). Histograms of fluorescence were registered over 512 channels with linear amplification. At least 8000 nuclei were analysed in each sample and the histogram mean value for G1 peaks evaluated using DPAC software (Partec). Calibration of the tetraploid *B. napus* at 200 resulted in the diploid *B. rapa* value between 85 and 90. Intermediate cytometric values were expected for putative triploid hybrids.

Male fertility (pollen viability)

Flowers collected from the field were placed on ice and pollen viability tests performed shortly afterwards. Pollen viability was inferred as the percentage of pollen stained by a 1% aceto-carmine solution. Approximately 100 pollen grains were counted per plant. Measures were repeated for questionable samples.

Amplified fragment length polymorphism – AFLPs

Genomic DNA was extracted from approximately 100 mg lyophilized material in a Fast Prep FP120 (BIO 101) grinder using a modified 2 × CTAB (cetyltrimethyl ammonium bromide) procedure. AFLPs were generated based on a modified protocol of Vos *et al*. (1995) as detailed in Halfhill *et al*. (2003). Five selective primer pair combinations were used: (i) *Eco*RI + AAC/*Mse*1 + CAC; (ii) *Eco*RI + AAC/*Mse*1 + CAG; (iii) *Eco*RI + AAG/*Mse*1 + CAA; (iv) *Eco*RI + AAG/*Mse*1 + CAT; and (v) *Eco*RI + AGG/*Mse*1 + CAC]. The amplified products were separated by PAGE in an automated sequencer (LI-COR) and infrared images were analysed.

All five primer pairs were used to analyse the hybrid generations for year 2003 and 2005 samples, but only primer pair no. 2 and 4 were used for year 2002 samples. AFLP markers were designated as *B. napus*-specific if they were absent in the *B. rapa* parents [QC-2975] and present in all individuals of *B. napus* (45A51) screened. The same criteria were used to define *B. rapa*-specific markers. A total of 45 *B. napus*-specific (7, 11, 8, 11, 8 for primer pairs 1–5, respectively,) and 32 *B. rapa*-specific AFLP markers (9, 10, 3, 6, and 4 for primer pairs 1–5) were scored. In 2002, a total of 25 *B. napus*- and 12 *B. rapa*-specific AFLP markers were scored. The presence/absence of each species-specific marker was recorded in all hybrids.

Results

We detected hybrid (defined here as including both F_1 and advanced generation hybrids) individuals in all 3 years at the St Henri site, and in 2003 at the Ste Agathe site (the *B. rapa* population observed on the field edge in 2003 was destroyed in 2005). Hybrid numbers decreased drastically over the 3-year period, from 85 out of ~200 plants surveyed in 2002 to only five out of 200 plants in 2005, 5 years after *B. rapa* was first in contact with the HR canola crop (St-Henri site) (Fig. 1, Table 2). HR *B. napus* volunteers were also evident at both sites. Both F_1 and backcross hybrid generations were detected. The HR trait was present in most of the hybrids over the 3-year period. Most hybrids had intermediate ploidy levels, i.e. intermediate flow cytometry levels, compared to values of 95 for *B. rapa* and 200 for *B. napus* (Table 3). The majority had values around 140, consistent with F_1 hybrids and a triploid genome (AAC) structure. Values other than an 'intermediate' flow cytometry value suggest backcross (BC) or advanced hybrid generations. A few individuals in 2002 had ploidy levels greater than that of *B. napus*, which may be due to production of unreduced gametes, a common occurrence in crosses involving self-incompatible *Brassica* species (Heyn 1977). Most hybrids had reduced pollen viability (~50% reduction), although a few had over 90% male fertility (Table 3). Fewer *B. rapa* (32) than *B. napus* (45) markers were found due to homology of the shared A genomes. Most hybrids had both species-specific markers. A large number of markers are needed to detect hybrids, and use of only two primer pairs in 2002 samples was not sufficient.

One introgressed individual, i.e. with the HR trait, diploid ploidy level of *B. rapa* and no AFLP *B. napus* markers, was observed from the St-Henri population in 2005 (Table 2). The latter (05-He-187) had reduced male fertility (i.e. 29% pollen viability), but did produce 1.55 g of seed, i.e. ~480 seeds (1000 seed wt = 3.2 g).

Seeds from this plant and that of two other putative/introgressed HR plants detected in 2005 were collected and grown in a growth chamber. Fifty seedlings were grown from plant 05-He-187; the shrivelled seeds from plant 05-He-47 did not germinate; and two seedlings were obtained from plant 05-He-141. Of the two offspring from 05-He-141, one was HR + and diploid, but scored positive for many *B. napus*-specific AFLP markers (Table 4). The progeny from plant 05-He-187 ($n = 50$) segregated 1:1 for the HR transgene (Fig. 1). Eighteen of the 22 HR+ offspring (82%) scored negative for *B. napus*-specific AFLP markers, whereas the other four offspring scored positive for only one or two *B. napus*-specific AFLP markers. All but two of

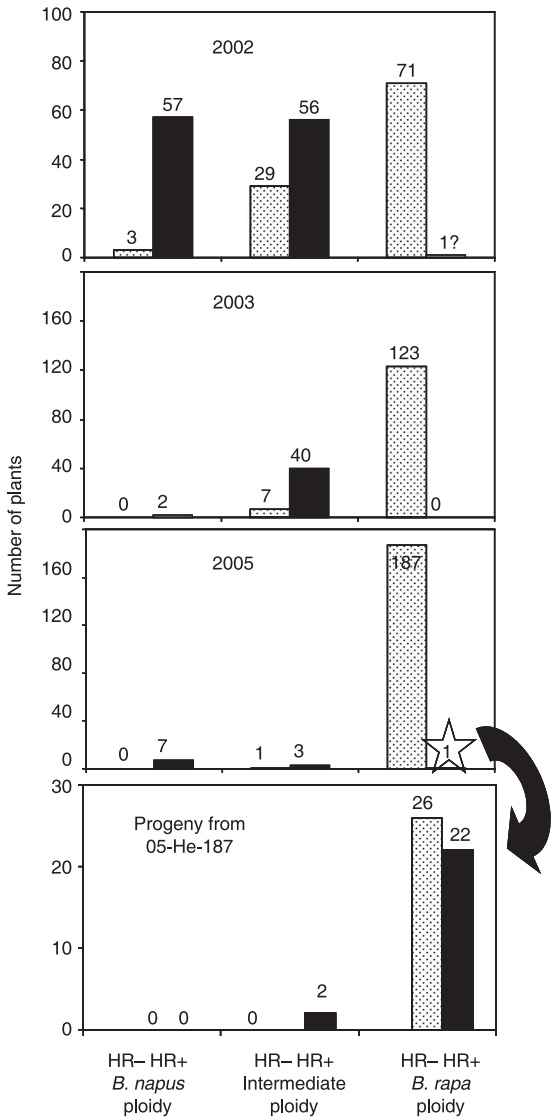

Fig. 1 Number of plants in a natural weed population with the herbicide resistant (HR+, solid bars) and susceptible (HR–, shaded bars) phenotypes detected at the St Henri site in 2002, 2003 and 2005, two, three, and five years, respectively, after *Brassica rapa* was first in contact with the HR canola crop: bars to the left: volunteer (plants originating from seed left from the crop) *Brassica napus* (tetraploid); bars in the centre: putative hybrids; bars to the right: weedy *B. rapa* (diploid) plants. Bottom graph: frequency of HR+ and HR– phenotypes and ploidy level in 50 progeny grown in a growth chamber from the introgressed diploid HR+ *B. rapa* individual (plant no. 05-He-187) detected in 2005.

the 50 offspring were diploid with high pollen viability (over 90%) (Table 4). Results from the progeny analysis provided evidence for introgression of the HR transgene.

Discussion

We have described a case where a transgene from an HR crop, after being introduced by gene flow into a weedy relative, persisted over a 6-year period in the absence of herbicide selection pressure (with the exception of possible exposure to glyphosate in 2002), and in spite of the fitness cost associated with hybridization (Halfhill *et al*. 2005; Warwick 2007). Although hybrid numbers dropped dramatically from 2002 to 2005, the HR transgene persisted in one of the two *B. rapa* populations. Transgene persistence was measured indirectly via molecular assessment of trait persistence using commercially available test strips; the link between this indirect molecular method and direct assessment of the presence of the transgene was very high as demonstrated in gene flow studies with glyphosate HR canola volunteers (Beckie *et al*. 2003). Evidence was also presented for the introgression of the HR transgene in *B. rapa* individuals. Although the introgressed plant had reduced fertility, its progeny had normal fertility, suggesting that it would likely have transmitted its transgenes into the local gene pool. However, complete confirmation of stable incorporation and evidence that copies of the transgene will remain in the weed population would still require the observation of several such naturally occurring transgenic plants with normal fertility.

Fitness of the different generations of hybrids is critical to the successful introgression of a transgene. It is important to distinguish between fitness costs and the benefits of the transgene per se, and the costs associated with interspecific hybridization. Hauser *et al*. (1998a) found that F_1 *B. rapa* × *B. napus* hybrids had intermediate fitness between the two parental species based on several combined characteristics, and they concluded that F_1 hybrids were significantly more fit than *B. rapa*. In a subsequent study, Hauser *et al*. (1998b) found that a fitness depression occurred in F_2 and backcrossed individuals, although a small percentage of hybrids were as fit as the parents. A comparison of introgressed transgenic glufosinate-resistant *B. rapa* × *B. napus* and nontransgenic BC_3 generation plants (Snow *et al.* 1999) indicated no fitness effects associated with the transgene, with pollen fertility and seed production of the BC_3 plants as great as those of *B. rapa* raised in the same growth rooms. In contrast, the fitness of two Canadian glyphosate-resistant *B. rapa* × *B. napus* BC_2F_2 backcross hybrid populations were less fit than the parental weed population, regardless of the presence/absence of the transgene, when grown under competitive field conditions in the absence of herbicide applications (Warwick 2007). Transgenic insect-resistant (Bt) *B. rapa* × *B. napus* hybrids with the insect-resistant transgene Bt-GFP [*Bacillus thuringiensis* (Bt)-green fluorescent protein (GFP)] also showed reduced fitness/competitiveness in various laboratory and field experiments (Halfhill *et al*. 2005). These studies mainly indicate reduced fitness in advanced backcross hybrid generations. However, our results suggest that such a potential fitness reduction, although it may have contributed to reduce hybrid numbers over time (Allainguillaume *et al*.

Table 2 Number (frequency in parentheses) of hybrid plant types found in two *Brassica rapa* × *B. napus* hybrid populations at two sites in Québec (Ste Agathe and St Henri) in 2002, 2003, and 2005

	Ste Agathe		St Henri		
Plant type	Year 2 2003	Year 4 2005*	Year 2 2002	Year 3 2003	Year 5 2005
B. napus volunteers (tetraploid and *B. napus* AFLPs)					
HR+	34 (49.3)	—	57 (23.1)	2 (1.28)	7 (3.5)
HR–	0	—	3 (1.22)	0	0
HR– (ploidy > *B. napus*)	0	—	2 (0.81)	0	0
B. rapa (diploid and *B. rapa* AFLPs)					
HR–	20 (29.0)	3 (100)	71 (28.7)	123 (78.3)	187 (94.0)
HR– (diploid?)	0	—	29 (11.7)	0	0
Triploid hybrids					
HR+ *B. rapa* and *B. napus* AFLPs Reduced fertility	3 (4.35)	—	23 (9.31)	15 (9.56)	3 (1.5)
HR+ *B. napus* AFLPs Similar/reduced fertility	8 (11.6)	—	14 (5.67)	14 (8.93)	0
HR+ *B. rapa* AFLPs	0	—	2 (0.81)	0	0
HR– *B. rapa* and *B. napus* AFLPs Reduced fertility	3 (4.35)	—	14 (5.67)	2 (1.28)	0
HR– *B. napus* AFLPs Reduced fertility	1 (1.45)	—	4 (1.62)	0	0
HR– *B. rapa* AFLPs Reduced fertility	0	—	2 (0.81)	1 (0.64)	1 (0.5)
Tetraploid or > tetraploid hybrids					
HR+ tetraploid *B. rapa* and *B. napus* AFLPs	0	—	12 (4.86)	0	0
HR+ > tetraploid *B. rapa* and *B. napus* AFLPs	0	—	2 (0.81)	0	0
Diploid hybrids					
HR+ *B. rapa* and *B. napus* AFLPs	0	—	2 (0.81)	0	0
HR– *B. rapa* and *B. napus* AFLPs	0	—	6 (2.43)	0	0
HR– *B. napus* AFLPs	0	—	3 (1.22)	0	0
Introgressed diploid plants					
HR+ *B. rapa* AFLPs	0	—	1 (0.41)	0	1 (0.5)
Summary					
Total number hybrids	15 (21.7)	0	85 (34.4)	32 (20.4)	5 (2.5)
Total number samples	69	3	247	157	199

*Field edge with *B. rapa* population was ploughed in 2005.

Table 3 Characterization of hybrid plants sampled from two *Brassica rapa* × *B. napus* hybrid populations at the St Henri and Ste Agathe sites, Québec and in 2001–2003, and 2005. Plants sampled in 2001 had been characterized previously (Warwick *et al*. 2003)

	No. of hybrids						
		HR trait					
Year	Total (*n*)	HR+	HR–	Cytometric value†	Pollen viability percentage	*B. rapa* AFLP markers (max: 32)	*B. napus* AFLP markers (max: 45)
						Mean (range)	
2001	63	63	0	141 (125–214)	55 (32–84)	11 (3–16)	43 (36–45)
2002	85	56	29	144 (82–232)	NA	2.4* (0–9)	15* (0–24)
2003	47	40	7	141 (87–181)	63 (0–98)	4 (0–15)	39 (0–45)
2005	5	4	1	144 (83–168)	46 (0–91)	10 (0–19)	16 (0–39)

*Only two of the five AFLP primer pairs were used on 2002 samples; *B. rapa* (max. 12); *B. napus* (max. 25).
†Calibration of the tetraploid *B. napus* at 200 resulted in the diploid *B. rapa* value between 85 and 90. Intermediate cytometric values were expected for putative triploid hybrids.
NA, not available.

Table 4 Progeny and hybrid characterization from two 2005 putative hybrid and introgressed plants (05-He-187 and 05-He-141) from the St Henri site

Ploidy	HR type	No. of progeny plants	Cytometric value*	Pollen viability percentage	*B. rapa* AFLP markers (max: 32)	*B. napus* AFLP markers (max: 45)
				Mean (range)		
05-He-187 (n = 50)						
Diploid	HR–	26	76 (69–85)	93 (55–100)	11 (8–16)	0.04 (0–1)
	HR+	22	77 (69–84)	90 (40–100)	9 (1–14)	0.2 (0–2)
Triploid	HR–	0	—	—	—	—
	HR+	2	143 (143–144)	95 (92–98)	9 (5–13)	0
05-He-141 (n = 2)						
Diploid	HR+	1	85	85	11	14
Triploid	HR+	1	109	54	11	36

*Calibration of the tetraploid *Brassica napus* at 200 resulted in the diploid *Brassica rapa* value between 85 and 90. Intermediate cytometric values were expected for putative triploid hybrids.

2006), did not prevent the successful introgression of a transgene into *B. rapa* under natural conditions. The process of transgene spread and introgression could be enhanced if the transgene were to convey fitness advantages to the hybrids, such as in the case of a Bt transgene that would cause release of herbivory pressure and thus improve fitness and survival of hybrids (Snow *et al*. 2003; Sutherland *et al*. 2006).

Although, *B. rapa* has a limited distribution as an agricultural and/or ruderal weed in *B. napus*-growing areas in Québec, transgene escape into natural wild populations of *B. rapa* via the formation of first generation hybrids has and will continue to occur when the two species grow sympatrically (Simard *et al*. 2006). The persistence of the HR trait over time in *B. rapa* populations will likely result from either seed bank longevity and/or continued F_1 hybrid production with *B. napus* volunteers. Volunteers can serve as a genetic bridge contributing to transgene persistence, as evidenced in *B. napus* (Hall *et al*. 2000) and *Helianthus annuus* (Reagon & Snow 2006). Introgression of the transgene into wild *B. rapa* plants should further contribute to the persistence and spread of the transgene, given that *B. rapa* is an obligate outcrossing species. We could also surmise that the potential for transgene stacking of multiple herbicide resistance traits via pollen-mediated gene flow in wild populations of *B. rapa* is high, as both glyphosate- and glufosinate-resistant F_1 *B. rapa* × *B. napus* hybrids occur within the same canola-growing region of Québec (Simard *et al*. 2006). In western Canadian crop fields, gene flow between different cultivars of *B. napus* quickly resulted in hybrid volunteers with multiple stacked resistance to herbicides (Hall *et al*. 2000; Beckie *et al*. 2003). There was no evidence of reduced fitness in multiple herbicide (glyphosate and glufosinate) resistant canola plants compared to single resistant plants under greenhouse conditions (Simard *et al*. 2005).

Introgression of the HR transgene in the *B. rapa* genome happened less than 6 years after the transgenic canola crop was grown. This process also happened in the absence of the selection pressure (the herbicide) during the last 3 years of monitoring, suggesting that selection for resistance could have been even quicker and/or more extensive, had the herbicide been used. Rapid transgene spread was documented recently for transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) in nonagronomic habitats only 2 years after a confined experimental trial released the transgene in the environment (Watrud *et al*. 2004; Reichman *et al*. 2006). The authors suggest that pollen-mediated gene flow, seed escape and herbicide use contributed to the spread of the transgene.

At present, there are no compelling data to suggest that the presence of an HR transgene in a wild or weedy relative is inherently risky. The actual consequences of hybridization and introgression will be trait-dependent, with some traits being more likely than others to increase weediness or invasiveness. It may also be event-specific depending on where the transgene is located and what other genes are introgressed along with the transgene. It is clear that in the case of herbicide resistance, the positive selective value of the trait will be restricted to habitats in the agro-ecosystem where the herbicide is applied. However, even in absence of selection pressure, the persistence of only a few fertile HR transgenic hybrids will ensure persistence of the transgenic trait over time, as was observed with *B. rapa* in Québec. These experiments show the importance of conducting risk assessment studies on transgenic hybrids under realistic agricultural/ecological conditions. This will be even more important for assessing the impact of fitness-enhancing traits, such as disease and insect resistance and stress tolerance to cold, drought, and salinity. These traits are not as well understood ecologically and clearly could have more impact if they were to spread to nonagricultural habitats.

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References

Allainguillaume J, Alexander M, Bullock JM *et al*. (2006) Fitness of hybrids between rapeseed (*Brassica napus*) and wild *Brassica rapa* in natural habitats. *Molecular Ecology*, **15**, 1175–1184.

Ammitzbøll H, Mikkelsen TN, Jørgensen RB (2005) Transgene expression and fitness of hybrids between GM oilseed rape and *Brassica rapa*. *Environmental Biosafety Research*, **4**, 3–12.

Beckie HJ, Harker KN, Hall LM *et al*. (2006) A decade of herbicide-resistant crops in Canada. *Canadian Journal of Plant Science*, **86**, 1243–1264.

Beckie HJ, Warwick SI, Nair H, Séguin-Swartz G (2003) Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). *Ecological Applications*, **13**, 1276–1294.

Chèvre A-M, Ammitzbøll H, Breckling B *et al*. (2004) A review on interspecific gene flow from oilseed rape to wild relatives. In: *Introgression from Genetically Modified Plants into Wild Relatives* (eds den Nijs HCM, Bartsch D, Sweet J), pp. 235–251. CABI Publishing, Wallingford, Oxfordshire, UK.

Dolezel J (1991) Flow cytometric analysis of nuclear content in higher plants. *Phytochemical Analysis*, **2**, 143–154.

Ellstrand NC (2001) When transgenes wander, should we worry? *Plant Physiology*, **125**, 1543–1545.

Ellstrand NC, Prentice HC, Hancock JF (1999) Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology and Systematics*, **30**, 539–563.

Halfhill MD, Millwood RJ, Raymer PL, Stewart CN Jr (2002) Bt-transgenic oilseed rape hybridization with its weedy relative, *Brassica rapa*. *Environmental Biosafety Research*, **1**, 19–28.

Halfhill MD, Millwood RJ, Weissinger AK, Warwick SI, Stewart CN Jr (2003) Additive transgene expression and genetic introgression in multiple GFP transgenic crop × weed hybrid generations. *Theoretical and Applied Genetics*, **107**, 1533–1540.

Halfhill MD, Richards HA, Mabon SA, Stewart CN Jr (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization and introgression with *Brassica rapa*. *Theoretical and Applied Genetics*, **103**, 362–368.

Halfhill MD, Sutherland JP, Moon HS *et al*. (2005) Growth, productivity, and competitiveness of introgressed weedy *Brassica rapa* hybrids selected for the presence of *Bt cry1*AC and *gfp* transgenes. *Molecular Ecology*, **14**, 3177–3189.

Halfhill MD, Zhu B, Warwick SI *et al*. (2004) Hybridization and backcrossing between transgenic oilseed rape and two related weed species under field conditions. *Environmental Biosafety Research*, **3**, 73–81.

Hall LM, Rahman MH, Gulden RH, Thomas AG (2005) Volunteer oilseed rape — will herbicide resistance traits assist ferality? In: *Crop Ferality and Volunteerism* (ed. Gressel J), pp. 59–79. CRC Press, London.

Hall L, Topinka K, Huffman J, Davis L, Good A (2000) Pollen flow between herbicide-resistant *Brassica napus* is the cause of multiple resistant *B. napus* volunteers. *Weed Science*, **48**, 688–694.

Hansen LB, Siegismund HR, Jørgensen RB (2001) Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* L. in a natural population. *Genetic Resources and Crop Evolution*, **48**, 621–627.

Hansen LB, Siegismund HR, Jørgensen RB (2003) Progressive introgression between *Brassica napus* (oilseed rape) and *B. rapa*. *Heredity*, **91**, 276–283.

Hauser T, Jørgensen R, Østergård H (1998b) Fitness of backcross and F_2 hybrids between weedy *Brassica rapa* and oilseed rape (*B. napus*). *Heredity*, **81**, 436–443.

Hauser T, Shaw RG, Østergård H (1998a) Fitness of F_1 hybrids between weedy *Brassica rapa* and oilseed rape (*B. napus*). *Heredity*, **81**, 429–435.

Hegde SG, Waines JG (2004) Hybridization and introgression between bread wheat and wild and weedy relatives in North America. *Crop Science*, **44**, 1145–1155.

Heyn FW (1977) Analysis of unreduced gametes in the Brassiceae by crosses between species and ploidy levels. *Zeitschrift für Pflanzenzüchtung*, **78**, 13–30.

James C (2006) *Global Status of Commercialized Biotech/GM Crops: 2006. ISAAA Briefs No. 35. ISAAA* (International Service for the Acquisition of Agri-Biotech Applications). Ithaca, New York.

Jørgensen RB, Andersen B (1994) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae): a risk of growing genetically modified oilseed rape. *American Journal of Botany*, **81**, 1620–1626.

Jørgensen RB, Andersen B, Landbo L, Mikkelsen TR (1996) Spontaneous hybridization between oilseed rape *(Brassica napus) and* weedy relatives. *Acta Horticulturae*, **407**, 193–200.

Landbo L, Andersen B, Jørgensen RB (1996) Natural hybridisation between oilseed rape and a wild relative: hybrids among seeds from weedy *B. campestris*. *Hereditas (Lund)*, **125**, 89–91.

Légère A (2005) Risks and consequences of gene flow from herbicide-resistant crops: canola (*Brassica napus* L.) as a case study. *Pest Management Science*, **61**, 292–300.

Linder CR, Taha I, Seiler GJ, Snow AA, Rieseberg LH (1998) Long-term introgression of crop genes into wild sunflower populations. *Theoretical and Applied Genetics*, **96**, 339–347.

Metz PLJ, Jacobsen E, Nap JP, Pereira A, Stiekema WJ (1997) The impact on biosafety of the phosphinothricin-tolerance transgene in inter-specific *B. rapa* × *B. napus* hybrids and their successive backcrosses. *Theoretical and Applied Genetics*, **95**, 442–450.

Mikkelsen TR, Jensen J, Jørgensen RB (1996) Inheritance of oilseed rape (*Brassica napus*) RAPD markers in a backcross progeny with *Brassica campestris*. *Theoretical and Applied Genetics*, **92**, 492–497.

Reagon M, Snow AA (2006) Cultivated *Helianthus annuus* (Asteraceae) volunteers as a genetic 'bridge' to weedy sunflower populations in North America. *American Journal of Botany*, **93**, 127–133.

Reichman JR, Watrud LS, Lee EH *et al*. (2006) Establishment of transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) in nonagronomic habitats. *Molecular Ecology*, **15**, 4243–4255.

Simard M-J, Légère A, Séguin-Swartz G, Nair H, Warwick S (2005) Fitness of double vs. single herbicide-resistant canola. *Weed Science*, **53**, 489–498.

Simard M-J, Légère A, Warwick SI (2006) Transgenic *Brassica napus* fields and *Brassica rapa* weeds in Québec: sympatry and weed-crop in situ hybridization. *Canadian Journal of Botany*, **84**, 1842–1851.

Snow AA (2002) Transgenic crops–why gene flow matters. *Nature Biotechnology*, **20**, 542.

Snow AA, Andersen B, Jørgensen RB (1999) Costs of transgenic herbicide resistance introgressed from *Brassica napus* into weedy *Brassica rapa*. *Molecular Ecology*, **8**, 605–615.

Snow AA, Pilson D, Rieseberg LH *et al*. (2003) A Bt transgene reduces herbivory and enhances fecundity in wild sunflowers. *Ecological Applications*, **13**, 279–286.

Stewart CN Jr, Halfhill MD, Warwick SI (2003) Transgene introgression from genetically modified crops to their wild relatives. *Nature Reviews Genetics*, **4**, 806–817.

Sutherland JP, Justinova L, Poppy GM (2006) The responses of crop-wild *Brassica* hybrids to simulated herbivory and interspecific competition: Implications for transgene introgression. *Environmental Biosafety Research*, **5**, 15–25.

Vos P, Hogers R, Bleeker M *et al*. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Warwick SI (2007) Gene flow between GM crops and related species in Canada. In: *The First Decade of Herbicide Resistant Crops in Canada. Topics in Canadian Weed Science*, **4** (eds Swanton C, Gulden R), pp. 101–113. Published by the Canadian Weed Science Society.

Warwick SI, Beckie HJ, Simard M-J, Légère A, Nair H, Séguin-Swartz G (2004) Environmental and agronomic consequences of herbicide-resistant (HR) canola in Canada. In: *Introgression from Genetically Modified Plants (GMP) Into Wild Relatives* (eds den Nijs HCM, Bartsch D, Sweet J), pp. 323–337. CABI Publishing, Wallingford, Oxfordshire, UK.

Warwick SI, Beckie HJ, Small E (1999) Transgenic crops: new weed problems for Canada? *Phytoprotection*, **80**, 71–84.

Warwick SI, Simard M-J, Légère A *et al*. (2003) Hybridization between transgenic *Brassica napus* L. and its wild relatives: *B. rapa* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L. and *Erucastrum gallicum* (Willd.) O.E. Schulz. *Theoretical and Applied Genetics*, **107**, 528–539.

Watrud LS, Lee EH, Fairbrother A *et al*. (2004) Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. *Proceedings of the National Academy of Sciences, USA*, **101**, 14533–14538.

Whitton J, Wolf DE, Arias DM, Snow AA, Rieseberg LH (1997) The persistence of cultivar alleles in wild populations of sunflowers five generations after hybridization. *Theoretical and Applied Genetics*, **95**, 33–40.

Wilkinson MJ, Elliott LJ, Allainguillaume J *et al*. (2003) Hybridization between *Brassica napus* and *B. rapa* on a national scale in the United Kingdom. *Science*, **302**, 457–459.

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